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Short communication

Effect of chronic administration of ethanol on $GABA_A$ receptor assemblies derived from α_2-, α_3-, β_2- and γ_2-subunits in the rat cerebral cortex

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Abstract

Chronic administration of ethanol decreased the immunoprecipitation of the [^{3}H]flunitrazepam binding activity for $GABA_A$ receptor assemblies derived from α_2-, α_3- and γ_2-subunits in the rat cerebral cortex. However, the [^{3}H]muscimol binding sites derived from these subunits were not affected. Thus, chronic ethanol causes the down-regulation of the benzodiazepine sites derived from the α_2-, α_3- and γ_2-subunits without affecting the GABA binding sites.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: GABA receptors

Keywords: Chronic ethanol; $GABA_A$ receptor; Subunit-specific antibody; Immunoprecipitation; Radioligand binding

The $GABA_A$ receptor is a transmembrane hetero-oligomeric protein comprised of pentameric assembly derived from a combination of various subunits, such as α_1–α_6, β_1–β_4, γ_1–γ_4, δ, ε, π and $\varnothing$ [2,11]. Although most of the native $GABA_A$ receptor assemblies consist of α-, β- and γ-subunits, the colocalization of these three major types of subunits is not an absolute requirement for the formation of the $GABA_A$ receptor [2,14,23]. The benzodiazepine site is localized at the interface of the α- and γ-subunits [25], and the GABA/muscimol binding site is localized at the interface of the α- and β-subunits [21,25]. The α_{2-5}-subunits are present mainly in the hippocampus and cerebral cortex, whereas the cerebellum is practically devoid of these subunits [11,24]. In contrast, the α_6-subunit is present in the cerebellar granule cells, whereas the hippocampus and cerebral cortex are devoid of this subunit [11,24]. The α_1-, β_2- and γ_2-subunits are present in abundant amount in almost every region of the brain [11,24]. Chronic administration of ethanol is reported to alter the mRNA and protein expression of various subunits of $GABA_A$ receptors [3–5,11,15–17,19,20,22]. However, these changes do not necessarily lead to similar changes in the assembly of subunits into mature $GABA_A$ receptors, since there are complex regulatory mechanisms of $GABA_A$ receptor expression at the transcriptional, translational, posttranslational and assembly levels, which vary with the subunit and brain area [6]. Although it is known that the chronic administration of ethanol reduces the α_1-subunit mRNA and polypeptide levels in the brain [4,5,17,19], we have demonstrated recently that chronic administration of ethanol does not result in a down-regulation of the $GABA_A$ receptor assemblies containing α_1-subunit [12]. In the present study, we have further investigated the effect of the chronic administration of ethanol on the $GABA_A$ receptor assemblies derived from the α_2-, α_3-, β_2- and γ_2-subunits in the rat cerebral cortex.

Adult male Sprague–Dawley rats weighing 225–250 g were obtained from Harlan (Indianapolis, IN, USA). All experiments were conducted in accordance with the Decla-

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ration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. Adequate measures were taken to minimize pain or discomfort to the animals. To demonstrate the effect of chronic administration of ethanol on the prevalence of the $GABA_A$ receptor assemblies, the rats were intoxicated by intragastric intubation method for 6 days, as described by us earlier [9,10,13]. Briefly, at the beginning of the experiment, a priming dose of ethanol of 5 g/kg (20% w/v) was administered orally to all animals. Following this, doses (9–15 g/kg over a 24-h period) were adjusted individually for each animal according to the presence or absence of ataxia and loss of righting reflex. Ethanol was administered as 20% w/v in normal saline three times a day for 6 days. Control rats received normal saline. Food and water were available ad libitum. There was no significant change in their body weight during the 6 days of the study. There was 10% mortality associated with this intragastric intubation method of ethanol administration. The ethanol-maintained rats were sacrificed 1 h after the last dose of ethanol. On the other hand, the ethanol-withdrawn rats were sacrificed 48 h after the last dose of ethanol. Cerebral cortices were dissected and stored at −80 °C until use. The rat cerebral cortex membrane preparation, the $GABA_A$ receptors solubilization, immunoprecipitation and radioligand binding assays were performed as described by us earlier [12]. Briefly, the tissue was thawed and homogenized in ice-cold 0.32 M sucrose solution (pH 7.4). The mixture was then centrifuged at 1000×*g* for 10 min at 4 °C, to discard the P_1 fraction. The supernatant was then centrifuged at 78,000×*g* for 30 min. The pellet was suspended in a 0.32 M sucrose solution (pH 7.4) and kept frozen overnight at −80 °C. After thawing, Tris–HCl (50 mM, pH 7.4) was added to the tissue, and it was centrifuged at 30,000×*g* for 10 min. The pellet was resuspended in Tris–HCl buffer (50 mM, pH 7.4) and centrifuged at 30,000×*g* for 10 min. The last step was repeated four more times. The pellet was resuspended in Tris–HCl buffer and kept frozen overnight at −80 °C. After thawing, Tris–HCl buffer was added to the tissue, and the mixture was centrifuged at 78,000×*g* for 30 min. The membranes were then suspended in Tris–HCl (50 mM, pH 7.4), distributed in aliquots and kept frozen at −80 °C until use. The $GABA_A$ receptors were solubilized in modified radioimmune precipitation assay buffer (RIPA), i.e., solubilization buffer (pH 7.4) containing NaCl (0.137 M), sodium deoxycholate (1% w/v), Triton X-100 (1% v/v), sodium dodecyl sulfate (SDS; 0.1% w/v), Tris (10 mM) and a cocktail of protease inhibitors containing EDTA (1 mM), EGTA (1 mM), benzamidine HCl (2 mM), trypsin inhibitor type 1-S (0.1 mg/ml), bacitracin (0.1 mg/ml) and phenylmethylsulfonyl fluoride (0.3 mM). After incubation for 1 h at 4 °C, insoluble material was removed by centrifugation at 100,000×*g* for 1 h. A sample of 400 μl (≈300 μg protein) of the solubilized receptors was incubated overnight at 4 °C with 20 μl of the subunit-specific anti-α_2 (amino acids 417–423; [18]), anti-α_3 (amino acids 1–13; [18]), anti-β_2 (amino acids 382–393; [8]) or anti-γ_2 (amino acids 1–29; [1]) antibody of the $GABA_A$ receptor, since our preliminary experiments revealed that 10–15 μl of these antibodies results in the maximal immunoprecipitation of the $GABA_A$ receptor assemblies. A little larger volume of antibodies (20 vs. 10–15 μl) was used to ensure complete immunoprecipitation. It did not increase the nonspecific binding, which was ≈10% for [^{3}H]flunitrazepam and ≈30% for [^{3}H]muscimol binding assays. The receptor–antibody complexes were recovered by incubation with protein A-agarose suspension (60 μl of 40% v/v), followed by centrifugation. Immunoprecipitation was quantified by determining the binding of various radioligands to the immunoprecipitated pellet and supernatant. Specific and nonspecific radioligand binding were determined with (i) [^{3}H]flunitrazepam (10 nM) vs. Ro 15-1788 (10 μM), and (ii) [^{3}H]muscimol (40 nM) vs. GABA (100 μM). Antibodies were procured from Alpha Diagnostic (San Antonio, TX, USA). The data are expressed as mean±S.E.M. The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett test.

The rat $GABA_A$ receptor α_2-, α_3-, β_2- and γ_2-subunits-specific antibodies immunoprecipitated 47.0±1.8%,

Table 1
Quantitative immunoprecipitation of the $GABA_A$ receptor assemblies by the antibodies specific for the α_2-, α_3-, β_2- and γ_2-subunits in the rat cerebral cortex

Group	% Immunoprecipitation of the binding activity			
	α_2-Subunit (*n*)	α_3-Subunit (*n*)	β_2-Subunit (*n*)	γ_2-Subunit (*n*)
[^{3}H]flunitrazepam (10 nM)				
Control	47.0±1.8 (6)	32.2±1.5 (7)	69.9±1.6 (5)	74.7±2.1 (5)
Ethanol dependent	37.8±1.6* (6)	25.5±0.6* (7)	74.9±2.4 (5)	66.9±1.1* (5)
Ethanol withdrawn	45.5±2.3 (6)	29.8±1.7 (7)	69.0±1.8 (5)	76.8±0.7 (5)
[^{3}H]muscimol (40 nM)				
Control	28.7±1.7 (6)	31.4±1.3 (5)	59.9±3.4 (4)	63.5±4.0 (3)
Ethanol dependent	29.5±1.8 (6)	31.0±1.1 (5)	66.6±3.8 (4)	69.4±3.1 (4)
Ethanol withdrawn	31.9±2.4 (6)	30.1±2.2 (5)	65.1±1.0 (4)	69.3±5.0 (4)

The values are mean±S.E.M. for the number of experiments indicated (*n*), each performed in triplicate. Immunoprecipitations were done using 20 μl of the antiserum specific for the rat α_2-, α_3-, β_2- or γ_2-subunit of the $GABA_A$ receptor. [^{3}H]flunitrazepam (10 nM) and [^{3}H]muscimol (40 nM) binding (100%) to the solubilized receptors (pellet+supernatant) of the rat cerebral cortex was 0.42±0.01 and 0.35±0.03 pmol/mg protein, respectively.

* $p<0.01$ as compared with the control group.